



**QUEEN'S
UNIVERSITY
BELFAST**

Fasciola hepatica

Hanna, R. E. B., Forster, F. I., Brennan, G. P., & Fairweather, I. (2013). Fasciola hepatica: Histological demonstration of apoptosis in the reproductive organs of flukes of triclabendazole-sensitive and triclabendazole-resistant isolates, and in field-derived flukes from triclabendazole-treated hosts, using in situ hybridisation to visualise endonuclease-generated DNA strand breaks. *Veterinary Parasitology*, 191(3-4), 240-251. <https://doi.org/10.1016/j.vetpar.2012.09.014>

Published in:
Veterinary Parasitology

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

Publisher rights

This is the author's version of a work that was accepted for publication in *Veterinary Parasitology*. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in *Veterinary Parasitology*, VOL 191, 01/2013

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Accepted Manuscript

Title: *Fasciola hepatica*: histological demonstration of apoptosis in the reproductive organs of flukes of triclabendazole-sensitive and triclabendazole-resistant isolates, and in field-derived flukes from triclabendazole-treated hosts, using *in situ* hybridization to visualise endonuclease-generated DNA strand breaks



Authors: R.E.B. Hanna, F.I. Forster, G.P. Brennan, I. Fairweather

PII: S0304-4017(12)00471-2
 DOI: doi:10.1016/j.vetpar.2012.09.014
 Reference: VETPAR 6514

To appear in: *Veterinary Parasitology*

Received date: 4-4-2012
Revised date: 5-9-2012
Accepted date: 6-9-2012

Please cite this article as: Hanna, R.E.B., Forster, F.I., Brennan, G.P., Fairweather, I., *Fasciola hepatica*: histological demonstration of apoptosis in the reproductive organs of flukes of triclabendazole-sensitive and triclabendazole-resistant isolates, and in field-derived flukes from triclabendazole-treated hosts, using *in situ* hybridization to visualise endonuclease-generated DNA strand breaks, *Veterinary Parasitology* (2010), doi:10.1016/j.vetpar.2012.09.014

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Fasciola hepatica: histological demonstration of apoptosis in the reproductive organs of flukes of triclabendazole-sensitive and triclabendazole-resistant isolates, and in field-derived flukes from triclabendazole-treated hosts, using *in situ* hybridization to visualise endonuclease-generated DNA strand breaks.

R.E.B. Hanna^{a*}, F. I. Forster^a, G.P. Brennan^b, I. Fairweather^b

^a *Veterinary Sciences Division, Agri-Food and Biosciences Institute, Stormont, Belfast BT4 3SD, United Kingdom*

^b *School of Biological Sciences, Queen's University, Belfast BT7 1NN, United Kingdom*

*Corresponding author. Tel.: +44 0 2890 525615;

fax: +44 0 2890 525767.

E-mail address: bob.hanna@afbini.gov.uk (R.E.B. Hanna).

ABSTRACT

Investigation of the triclabendazole (TCBZ) resistance status of populations of *Fasciola hepatica* in field cases of fasciolosis, where treatment failure has been reported, can be supported by histological examination of flukes collected from recently treated hosts. In TCBZ-sensitive flukes (TCBZ-S) exposed to TCBZ metabolites for 1-4d *in vivo*, but not in TCBZ-resistant flukes (TCBZ-R), morphological changes suggestive of apoptosis occur in cells undergoing meiosis or mitosis in the testis, ovary and vitelline follicles. In order to verify or refute the contention that efficacy of TCBZ treatment is associated with apoptosis in the reproductive organs of flukes, histological sections of TCBZ-S (Cullompton isolate) flukes and TCBZ-R (Sligo isolate) flukes were subjected to the TdT-mediated dUDP nick end labelling (TUNEL) *in situ* hybridization method, a commercially-available test specifically designed to label endonuclease-induced DNA strand breaks associated with apoptosis. Additionally, sections of *in vivo*-treated and untreated flukes

originating from field outbreaks of suspected TCBZ-S and TCBZ-R fasciolosis were labelled by the TUNEL method. It was found that in treated TCBZ-S flukes, strong positive labelling indicating apoptosis was associated with morphologically abnormal cells undergoing mitosis or meiosis in the testis, ovary and vitelline follicles. Background labelling in the positive testis sections was attributed to heterophagy of cell debris by the sustentacular tissue. The triggering of apoptosis was probably related to failure of spindle formation at cell division, supporting the contention that TCBZ inhibits microtubule formation. In treated TCBZ-R (Sligo type 1) flukes, and in treated flukes from field outbreaks of suspected TCBZ-R fasciolosis, no significant labelling was observed, while sections of fluke derived from a field case of fasciolosis where TCBZ resistance was not suspected were heavily labelled. Light labelling was associated with the testis of untreated Cullompton (TCBZ-S) and Sligo type 2 (TCBZ-R) flukes, which exhibit abnormal spermatogenesis and spermiogenesis, respectively. This was attributed to apoptosis and to heterophagy of effete germ line cells by the sustentacular tissue. It is concluded that demonstration of apoptosis by *in situ* hybridisation using the TUNEL method on sections of 1-4 d *in vivo* TCBZ-treated *F. hepatica* can contribute to the diagnosis of TCBZ resistance in field outbreaks of fasciolosis.

Keywords: *Fasciola hepatica*; triclabendazole-sensitive and –resistant isolates; reproductive organs; *in situ* hybridisation; histology; apoptosis.

1. Introduction

The benzimidazole anthelmintic triclabendazole (TCBZ) was introduced as a veterinary fasciolicide in 1983 and, because of its broad spectrum of activity against liver fluke (*Fasciola* spp.) of all ages down to 2 days post-infection in the definitive host (Boray et al., 1983), it rapidly gained widespread use for the treatment and control of both chronic and acute fasciolosis in ruminant livestock throughout the world. However, as a result of this extensive use, fluke resistance to TCBZ began to emerge, and was first documented in

67 Australia (Overend and Bowen, 1995). Since then, cases of resistance have
 68 been reported in Europe and in South America (Fairweather, 2005, 2009,
 69 2011a; Olaechea et al., 2011). While anecdotal reports of failure of efficacy of
 70 TCBZ in the field continue to accumulate, and farmers are switching from use
 71 of TCBZ to other flukicides which are less effective against the juvenile and
 72 immature stages, there is debate regarding the criteria on which diagnosis of
 73 TCBZ resistance should be based (Fairweather, 2011b, c). The TCBZ
 74 resistance status of fluke isolates can be established unequivocally using
 75 appropriately designed clinical trials (McConville et al., 2009; Fairweather,
 76 2011a, b, c; Flanagan et al., 2011a, b), but they are time-consuming and
 77 expensive to run. Faecal egg count reduction tests (FECRT) can reveal failure
 78 of anthelmintic treatment and, if backed up by supplementary tests such as
 79 the coproantigen reduction test (CRT) (Flanagan et al., 2011a, b), post-
 80 treatment fluke histopathology (Hanna et al., 2010, 2012a) and egg hatch
 81 (Fairweather et al., 2012), can give a relatively rapid and economic appraisal
 82 of the likely TCBZ resistance status of field isolates. In particular, examination
 83 of the reproductive tract of flukes from recently-treated sheep on individual
 84 farms can reveal the presence or absence of histological changes consistent
 85 with TCBZ action. Even as early as 24 h after administration of TCBZ to
 86 experimentally infected sheep, the formation of normal shelled eggs in the
 87 ootype of TCBZ-sensitive (TCBZ-S), but not of TCBZ-resistant (TCBZ-R)
 88 flukes, is disrupted, with appearance of abnormal contents in the proximal
 89 coils of the uterus (Hanna et al., 2012a). Such information is of practical
 90 importance, in that it provides a rational basis for provision of advice to flock
 91 managers regarding future choice of flukicide and appropriate management of
 92 dosing regimes. Conspicuous amongst the histological changes that develop
 93 in TCBZ-S flukes following *in vivo* exposure to TCBZ metabolites are
 94 morphological features suggestive of apoptosis in cells undergoing mitosis or
 95 meiosis (spermatogonia and spermatocytes in the testis; oogonia and primary
 96 oocytes in the ovary; and stem cells in the vitelline follicles) (Kumar et al.,
 97 2005; Hanna et al., 2010; Toner et al., 2011a, b). In the present study, *in situ*
 98 hybridisation was used to investigate whether the morphological changes
 99 documented in histological sections of affected fluke testis indeed coincide

with endogenously-triggered individual cell death. Potentially, investigation of the TCBZ resistance status in field populations of *F. hepatica* using FECRT or CRT, could be supported, but for economic reasons not replaced, by immunocytochemical methods to demonstrate apoptosis in the reproductive organs of *in vivo*-exposed flukes.

2. Methods and Materials

2.1. Source of flukes

2.1.1. Experimental trial

Flukes used in this study were collected in the course of a previously reported experimental trial to study histological changes in the reproductive organs of TCBZ-S and TCBZ-R flukes induced by *in vivo* treatment of infected sheep with TCBZ (Hanna et al., 2010). Briefly, 10 shed-reared Blackface X sheep (4-5 months old), checked for the absence of helminth infection by faecal examination, were subsequently infected by oral gavage with 250 metacercariae obtained from laboratory colonies of *Galba truncatula* maintained at Queen's University, Belfast, Northern Ireland. Six of the sheep received metacercariae of the TCBZ-S Cullompton fluke isolate and 4 received metacercariae of the TCBZ-R Sligo fluke isolate. Information on the provenance and TCBZ-sensitivity of these isolates was reviewed by Fairweather (2011a). Twelve weeks after infection, 4 of the TCBZ-S infected sheep were dosed with 10 mg/kg TCBZ (Hennessy et al., 1987). These sheep were slaughtered humanely by exsanguination following captive bolt stunning 48 h or 72 h after treatment, the livers were removed and all flukes present in the bile ducts and gall bladders were collected in warm (37°C) 0.9% (w/v) NaCl. The remaining 2 TCBZ-S infected sheep were not treated with anthelmintic, but were slaughtered 24 h after the other sheep had been dosed, and the flukes from their livers were collected to provide untreated control material. Of the 4 sheep that were infected with the TCBZ-R fluke isolate, two were dosed with TCBZ (10 mg/kg; Hennessy et al., 1987) and slaughtered 48 h later for fluke collection. The remaining 2 sheep were not treated with anthelmintic, but were slaughtered 24 h after the other sheep

131 were dosed, to provide untreated flukes for control material. The rational of
132 dose-to-slaughter time was discussed previously (Hanna et al., 2010).

133 2.1.2. *Field cases with suspected TCBZ- resistance*

134 Rectal faeces samples were collected individually from 5-10 sheep
135 assembled on each of 3 widely-separated farms in Northern Ireland where
136 TCBZ resistance was independently suspected (on the basis of previous
137 treatment failure). These animals were individually dosed to weight with TCBZ
138 (10mg/kg). Seventy-two h later, on confirmation of a positive pre-treatment
139 FEC and coproantigen result (using the protocols described by Flanagan et
140 al., 2011a, b), one animal from each group was slaughtered humanely by
141 exsanguination following captive bolt stunning. The liver was removed and all
142 flukes present in the bile ducts and gall bladder were collected in warm (37°C)
143 0.9% (w/v) NaCl. In order to confirm failure of TCBZ treatment on each of the
144 farms, rectal faeces samples were collected from the sheep remaining in each
145 group 3 weeks after TCBZ treatment, and post-treatment FECs and
146 coproantigen levels were determined.

147 2.1.3. *Rats infected with metacercariae from bovine field case*

148 Approximately 150 specimens of *Galba truncatula* were collected from wet
149 pasture on a dairy farm where fasciolosis had been diagnosed in the cattle in
150 each of several successive years, but triclabendazole had not been used.
151 These snails were induced to shed metacercariae, by immersing them in
152 clean water chilled to 10°C. Five metacercariae were delivered by oral gavage
153 to each of a group of 6 male Wistar rats. Twelve weeks after infection, the rats
154 were dosed orally with 10mg/kg TCBZ (Devine et al., 2011) and 48h later they
155 were euthanized using CO₂, prior to collection of flukes from the main bile
156 duct of each animal. From each rat, between 1 and 4 flukes (mean 2.5) were
157 recovered.

158 2.2. *Preparation of flukes for histology*

159 Fifteen flukes were collected from each sheep, and all the flukes from the
160 infected and treated rats were fixed for histological examination less than 30
161 min after removal from the host. The flukes were placed in a 10 cm-square

162 plastic Petri dish and held flat beneath a light glass plate throughout fixation
 163 for 24 h with 10% (v/v) neutral-buffered formalin. After fixation, each fluke was
 164 sliced into equal right and left halves along the median plane. The two halves
 165 were dehydrated in ethanol, cleared in Clearene (Surgipath Europe Ltd.) and
 166 embedded in a wax block following conventional procedures, with the cut
 167 surfaces presented at the block face. Sections 5µm thick were cut from each
 168 block face and stained with haematoxylin and eosin (H&E). These sections
 169 were used to identify histological changes consistent with apoptosis in the
 170 treated TCBZ-S flukes, as described by Hanna et al. (2010). Additional
 171 sections were cut from 5 wax blocks in each batch of 15 blocks derived from a
 172 single sheep. These sections were used in an *in situ* hybridization method to
 173 demonstrate the occurrence and distribution of apoptosis, as described below.
 174 All sections were examined and photographed using a Leica DM LB2
 175 microscope with a Nikon Coolpix 5000 camera system.

176 2.3. *In situ hybridization method to demonstrate apoptosis*

177 The TUNEL (TdT-mediated dUDP nick end labelling) *in situ* hybridization
 178 method, designed to specifically label endonuclease-induced DNA strand
 179 breaks associated with apoptosis, was carried out on sections of *in vivo*
 180 TCBZ-treated and untreated control TCBZ-S and TCBZ-R flukes using a
 181 commercially available kit (*In situ* Cell Death Detection POD kit,
 182 Cat.No.1684817, Roche Diagnostics GmbH, Roche Molecular Biochemicals,
 183 Mannheim, Germany).

184 The procedure used was based on that described in the Instruction Manual.
 185 Sections of the formalin-fixed flukes were de-waxed, rehydrated and
 186 equilibrated in 5mM Tris-buffered saline (pH 7.6)(TBS). Endogenous
 187 peroxidases were blocked by incubation at room temperature in 0.5% (v/v)
 188 H₂O₂ in methanol for 20 min, following which the sections were again washed
 189 in TBS prior to incubation for 15 min at 37°C in proteinase K (25µg/ml in TBS)
 190 for antigen retrieval. Further TBS washing was followed by permeabilisation
 191 using 0.1% (v/v) Triton X in 0.1% (w/v) sodium citrate (2 min, 4°C). After
 192 further TBS washing, 50µl of TUNEL reaction mixture (containing TdT and
 193 fluorescein-labelled nucleotides) was applied to each section, for an

incubation period of 60 min at 37°C. Subsequent washing with 1% (v/v) bovine serum albumin (BSA) in TBS was followed by application of 50µl Converter-POD (anti-fluorescein antibody conjugated with horse-radish peroxidase) for an incubation period of 30 min at 37°C. After further washing with TBS + 1% BSA, diaminobenzidine (DAB) substrate for POD (Peroxidase Substrate kit DAB, Cat.No. SK-4100, Vector Laboratories Inc., Burlingame, CA, USA) was applied to the sections for 5-7 min. Final TBS washing of the sections was followed by counterstaining using Harris's haematoxylin, and the sections were blued, dehydrated, cleared and mounted following conventional histological procedures. Negative control sections were prepared with each labelling batch. These sections were incubated with TUNEL reaction mixture from which the TdT was omitted. Positive controls were also included. To prepare them, TACS nuclease (TACS.XL DAB *In situ* Apoptosis Detection kit, Cat. No. 4828-30-DK, Trevigen Inc., Gaithersburg, MD, USA) was included in the TUNEL reaction mix. This exogenously applied nuclease generates DNA strand breaks in all the nuclei exposed in the tissue section.

3. Results

3.1. TCBZ-S (*Cullompton isolate*) flukes

3.1.1. Testis

In this triploid fluke isolate, spermatogenesis is arrested at the primary spermatocyte stage. In untreated flukes, primary and secondary spermatogonia occurred in clusters towards the periphery of the tubules, while tertiary spermatogonia, the most abundant cell type present, mainly occupied the central area. Secondary spermatocytes, spermatids and spermatozoa were not represented in the testis tubules (Fig. 1a). Primary spermatocytes often appeared irregular, with pyknotic or karyorrhectic nuclei and eosinophilic cytoplasm, and were sometimes represented by large rounded eosinophilic bodies containing dense irregular masses of chromatin (Fig. 1a). In sections of untreated *Cullompton isolate* flukes that had been subjected to TUNEL *in situ* hybridization, mild positive labelling was sometimes seen in the primary spermatocytes and in the 'apoptotic-like' eosinophilic bodies, especially towards the periphery of the profiles, but rarely elsewhere in the testis tubules

(Fig. 1b). Sections incubated with TUNEL reaction mixture from which the TdT was omitted displayed no evidence of reaction product ('negative control sections') (Fig. 1c), while nuclei of cells in all tissues of the sections incubated in the presence of exogenous TACS nuclease were intensely labelled ('positive control sections') (Fig. 1d).

In TCBZ-S Cullompton isolate flukes that had been exposed *in vivo* to TCBZ for 2 or 3 days, the testis profiles in H&E-stained sections were markedly depleted of cellular content, displaying marked peripheral vacuolation and an increase in cell-free space, which nevertheless stained lightly with eosin, indicating the presence of protein-containing fluid (Fig. 2a). While primary and secondary spermatogonia often remained recognisable towards the periphery of the tubules, tertiary spermatogonia were particularly reduced in number. Amongst the cells that remained in the profiles, eosinophilic rounded primary spermatocytes were particularly abundant and many mononuclear cells, presumably spermatogonia, also displayed pyknotic nuclei and eosinophilic cytoplasm. The changes were more pronounced in the 3 day-treated flukes than in the 2 day-treated samples and, in the former, few of the remaining cells were recognisable.

In sections of 2 and 3 day TCBZ-treated Cullompton flukes, on which TUNEL *in situ* hybridization had been carried out, strong positive labelling was evident throughout the testis profiles. The rounded eosinophilic primary spermatocyte rosettes and many of the pyknotic mononuclear cells displayed dense labelling, particularly those located in the peripheral vacuoles, while the cell-free fluid within the profiles showed moderate intensity of reaction (Fig. 2b). In negative control sections of TCBZ-treated Cullompton flukes, no labelling was seen, as was the case with the negative control sections of untreated flukes (Fig.1c).

3.1.2. Ovary

In untreated flukes, oogonia, each with a heterochromatic nucleus and a thin layer of basophilic cytoplasm, occurred peripherally in the thick-walled muscular ovarian tubules, while rounded or polygonal primary oocytes with abundant cytoplasm and relatively euchromatic nuclei bearing 1 or 2 nucleoli, were densely packed in the core of each tubule (Fig. 2c). In sections that had

been subjected to TUNEL *in situ* hybridization, light background labelling was sometimes seen over a few oogonia, but in general the profiles were free of label (Fig. 2c). As with the testis, negative control sections displayed no labelling, whilst all nuclei in the positive control sections were intensely labelled.

In sections of ovary of TCBZ-treated flukes there was a progressive loss of cells that was especially evident after 72 h treatment. Oogonia and oocytes often appeared shrunken and rounded with pyknotic nuclei and intensely eosinophilic cytoplasm. The tubules were reduced in diameter compared to those of the untreated flukes, and sometimes irregular empty spaces and vacuoles appeared in the lumen. In sections treated by the TUNEL method, densely-labelled single cells corresponding to apoptotic oogonia and oocytes were evident in the peripheral and core regions, respectively, of the tubules (Fig. 2d).

3.1.3. Vitelline follicles

In untreated flukes, vitelline cells at all stages of development were recognisable, from stem cells, and intermediate cells located towards the periphery of the follicles to the bulging, more centrally-located mature cells. Stem cells had scant basophilic cytoplasm lacking inclusions, while intermediate cells featured refringent globules of shell precursor protein in the cytoplasm. In mature cells, the cytoplasmic volume was expanded and achromic, while the clusters of shell precursor protein globules were marginalised. In sections subjected to the TUNEL method, no labelling was detected in the vitelline follicles, indicating absence of endonuclease-induced DNA strand breaks in this tissue (Fig. 3a). In flukes treated *in vivo* with TCBZ for 48 and 72 h, there was progressive cellular depopulation of the vitelline follicles with corresponding shrinkage, and this was particularly evident after 72 h treatment. The numbers of stem cells and intermediate cells were reduced, while the cells remaining were predominantly of the mature type. They often appeared to be breaking down, and shell protein globules were irregular in size, density and distribution. At the periphery of the follicles, many stem cells and intermediate vitelline cells appeared pyknotic or karyorrhectic, with eosinophilic cytoplasm, and vacuolation was often noted. In sections

treated with the TUNEL reaction mixture, single-cell labelling was evident at the periphery of many vitelline follicles, sometimes with strands of less intense reactivity extending circumferentially (Fig. 3b).

3.1.4. Other tissues

In the TCBZ-treated flukes, as compared to untreated flukes, the cytoplasm of both types of Mehlis' gland secretory cells, S1 and S2, appeared shrunken, vesiculated and lacking secretory bodies. Their elongated cytoplasmic connections to the ootype were vacuolated, as was the supporting parenchymal matrix. In sections subjected to the TUNEL procedure, no labelling was seen in the Mehlis' gland of flukes exposed to TCBZ for 48h or 72h (Fig. 3c), which was similar to the situation in untreated flukes. The uterine coils of the TCBZ-treated flukes (Fig. 3d) lacked the shelled eggs characteristic of untreated flukes (Fig. 3e). In 48 h-treated flukes, the uteri contained only clusters of vitelline cells that lacked shell protein globules, together with occasional free ova and irregular masses of hyaline shell protein, whilst in 72 h-treated flukes the uteri displayed sparse content. In sections treated by the TUNEL method, no labelling was seen to be associated with the cellular or amorphous content in the uterus of treated flukes, or with the eggs in untreated flukes. However, the natural yellow-brown colour of the tanned shell protein masses (Fig. 3d) and eggs shells (Fig. 3e) somewhat resembled light positive staining.

The integrity of the gastrodermis and tegumental syncytium frequently appeared disrupted in TCBZ-treated flukes, particularly those exposed to the anthelmintic for 72 h. Often in these cases the parenchymal tissue appeared vacuolated and 'open' in texture. In TUNEL-treated sections of untreated and TCBZ-treated flukes, the nuclei of the tegumental perikarya, gut and parenchyma were found to be unlabelled.

3.2. TCBZ-R (*Sligo isolate*) flukes

3.2.1. Testis

Approximately half of the flukes (*Sligo* Type 1) showed all stages of spermatogenesis and spermiogenesis, and mature sperm were evident within the testis profiles. The remaining flukes (*Sligo* Type 2) exhibited all stages of

spermatogenesis up to the spermatid stage, but no mature spermatozoa were present in the testes. No differences in testis histology were seen for either type of fluke when 48h and 72h TCBZ-treated individuals were compared with untreated controls. In sections of untreated and TCBZ-treated flukes that had been subjected to the TUNEL method for demonstration of endonuclease-generated DNA strand breaks, no labelling was seen in the Type 1 flukes (Fig. 4a). In many of the Type 2 flukes, heterogeneous, irregularly-distributed, granular positive labelling was noted around the periphery of some testis profiles, often in the peripheral vacuoles (Fig. 4b). This type of labelling was found in both the untreated and the 48h and 72h TCBZ-treated flukes. Negative control sections were unlabelled, whilst in positive control sections, all nuclei were densely labelled.

3.2.2. Ovary

The ovarian tubules of untreated TCBZ-R Sligo isolate flukes, and also of 48h and 72h *in vivo* TCBZ-treated TCBZ-R flukes, displayed histological features similar to those of the untreated TCBZ-S flukes described above. The branches of the ovary in all the flukes examined were densely packed with peripheral oogonia and central oocytes, and no lesions associated with flukicide action were recognised. In TUNEL-treated sections, no labelling was seen.

3.2.3. Vitelline follicles

The vitelline follicles of untreated TCBZ-R Sligo isolate flukes and of those exposed to TCBZ for 48h or 72h *in vivo* displayed histological features similar to the untreated TCBZ-S flukes described above. The proportions of stem cells, intermediate cells and mature cells were as in the untreated TCBZ-S flukes, and the mature cells retained intact and distinct cell boundaries. No lesions were seen that could be attributed to TCBZ action and, in the sections that were subjected to the TUNEL method, no labelling indicative of apoptosis was seen.

3.2.4. Other tissues

In untreated TCBZ-R Sligo isolate flukes and those exposed to TCBZ *in vivo* for 48h or 72h, no significant differences were seen from the untreated

TCBZ-S Cullompton flukes in the histology of the Mehlis' gland, tegument, gut or parenchymal tissue. Numerous well-shelled eggs and mature spermatozoa were present in the uterus of treated and untreated Sligo isolate flukes. No changes were seen that might be attributed to TCBZ action and, in sections subjected to the TUNEL procedure, no labelling indicative of apoptosis was seen.

3.3. *Flukes from field cases with suspected TCBZ-resistance*

Comparison of pre-dosing and 3-week post-dosing FECs and coproantigen analysis results in the sheep sampled from each of the three individual flocks in which TCBZ resistance was suspected revealed no significant differences, supporting the flockowners' contention of resistance status. In all of the flukes examined from each of the three sheep slaughtered 72h post-TCBZ treatment, the histological features of the reproductive and somatic tissues closely resembled those of untreated Sligo (TCBZ-R) Type 1 flukes (Section 3.2). In profiles of testis, all stages of spermatogenesis and spermiogenesis (including primary, secondary and tertiary spermatogonia, primary and secondary spermatocytes, spermatids and mature spermatozoa) were represented. In sections subjected to the TUNEL procedure, no labelling was seen in the testis, ovary, vitelline follicles, Mehlis' gland or in any of the somatic tissues. Negative control sections also lacked labelling, while in the positive control sections, all nuclei were labelled, as anticipated.

3.4 *Flukes from rats infected with metacercariae from bovine field case*

All of the flukes collected from rats 48h after TCBZ treatment showed histological changes in the testis, ovary, vitelline cells, Mehlis' gland and uterus that were consistent with full efficacy of drug action, when compared with sections of Cullompton (TCBZ-S) flukes from 48h and 72h TCBZ-treated sheep (Section 3.1). In the testis profiles, there was marked depletion of cellular content, with peripheral vacuolation, accumulation of eosinophilic fluid and presence of numerous rounded primary spermatocytes and mononuclear cells displaying pyknotic nuclei and eosinophilic cytoplasm. Unlike flukes of the Cullompton isolate, spermatids and mature spermatozoa also featured in

the testis tissue of these flukes. In sections on which TUNEL *in situ* hybridization had been carried out, strong positive labelling was seen throughout the testis profiles. The rounded primary spermatocytes and the pyknotic mononuclear cells often displayed dense labelling, particularly those cells associated with the peripheral vacuoles, while the cell-free fluid contents also showed mild to moderate intensity of reaction (Fig. 4c). In sections of ovary stained with H&E, there was a reduction in cellular content, with oogonia and oocytes often appearing shrunken and rounded, and displaying pyknotic nuclei and intensely eosinophilic cytoplasm. In TUNEL-treated sections, generally there was intense labelling of these rounded cells in the periphery and core of the ovarian tubules (Fig. 4d). In the vitelline follicles there was shrinkage, with a reduction in numbers of stem cells and intermediate-type vitelline cells, and a relative increase in the number of mature-type cells, as was the case in TCBZ-treated Cullompton flukes (Section 3.1.3). At the periphery of the follicles, cells often appeared pyknotic or karyorrhectic and, in sections treated with the TUNEL reaction mixture, these rather isolated abnormal cells were seen to be densely labelled (Fig. 4e). The Mehlis' gland complex of these flukes from TCBZ-treated rats displayed vesiculation and shrinkage, while in the uterine coils no shelled eggs were seen, but free vitelline cells and amorphous masses of shell protein were present. No significant labelling was noted in the TUNEL-treated sections. These findings paralleled the situation found in equivalent tissues of Cullompton flukes exposed to TCBZ *in vivo* (Section 3.1.4).

4. Discussion

Programmed cell death or apoptosis is characterised by nuclear collapse with extensive damage to chromatin and cleavage of DNA into oligonucleosomal length fragments after activation of a calcium-dependant endogenous endonuclease (Duvall and Wyllie, 1986; Compton, 1992). Endonucleolysis is considered to be the key biochemical event of apoptosis, and the induced DNA strand breaks can be localised in histological preparations using an *in situ* method that involves incorporation of labelled nucleotides at the strand break sites using terminal deoxynucleotidyl

transferase (TdT) (Gavrieli et al., 1992). This so-called TUNEL (TdT-mediated dUDP nick end labelling) technique is highly sensitive for endonuclease-induced strand breaks, and specifically discriminates apoptosis from necrosis and primary DNA strand breaks, such as might be induced by drug action (Gorczyca et al., 1993; Gold et al., 1994). Oligonucleosomal DNA cleavage is generally, but not invariably, accompanied by morphological changes associated with apoptosis, such as pyknosis, karyorrhexis and cytoplasmic eosinophilia (Cohen et al., 1992).

In previous studies on the histology of TCBZ-S isolates of *F. hepatica*, subjected to TCBZ treatment *in vivo* (Hanna et al., 2010, 2012a; Toner et al., 2011a, b), morphological changes consistent with apoptosis were described in certain cell types in the reproductive organs. Specifically, many of those cells that normally undergo mitosis or meiosis, (the spermatogonia and spermatocytes in the testis, the oogonia and oocytes in the ovary, and the stem cells in the vitelline follicles), become rounded or partially isolated from surrounding tissues, developing pyknotic or karyorrhectic nuclei and eosinophilic cytoplasm. At the same time, the cell populations in these organs declines due to a failure to replace the maturing cell types (spermatids and spermatozoa, primary oocytes and mature vitelline cells in the testis, ovary and vitelline follicles, respectively), with new cells generated by cell division at the periphery of the organs. The triggering of apoptosis in these dividing cells has been attributed to failure of spindle formation during mitosis or the first meiotic division, a result of inhibition of microtubule formation by TCBZ. The activity of the benzimidazole-class anthelmintics, to which TCBZ belongs, is believed to lie in their ability to bind β -tubulin, thus inhibiting microtubule-mediated processes such as spindle formation (Lacey, 1988; Fairweather, 2005, 2009). In the present study, many of the cells showing morphological changes associated with apoptosis in the testis, ovary and vitelline follicles of TCBZ-S flukes exposed to metabolites of the drug *in vivo*, gave a strong positive signal with the TUNEL labelling method. This confirms the occurrence of endonuclease-induced DNA strand breaks in these abnormal cells, and supports the concept that TCBZ activity targets spindle formation in dividing cells, triggering the cascade of events that leads to internally-programmed cell

death. This is a process that serves to eliminate cells that are irreparably damaged, particularly if that damage affects the DNA (Kumar et al., 2005).

It was noted that occasional abnormal primary spermatocytes in untreated Cullompton (TCBZ-S) flukes, particularly those close to the periphery of the tubules, also gave positive labelling with the TUNEL method. This is consistent with triploidy in flukes of this isolate, leading to failure of segregation at the first meiotic division in spermatogenesis and triggering apoptosis in the primary spermatocytes (Fletcher et al., 2004; Hanna et al., 2008). In the TCBZ-R Sligo isolate, approximately 50% of the flukes exhibit the abnormal Type 2 testis, in which the spermatids fail to develop to mature spermatozoa (Hanna et al., 2008). Here, irregular positive TUNEL labelling was noted in the peripheral zone of the testis tubules, probably corresponding with endonuclease-generated degradation of effete spermatid nuclei. The testis tubules of the Sligo Type 1 flukes, in which spermatogenesis and spermiogenesis continue to completion, with production of normal-appearing spermatozoa, were unlabelled. Recently, it has been shown that sustentacular tissue is located in the peripheral zone of the testis tubules in *F. hepatica* (Hanna et al., 2012b in press). A primary function of this tissue appears to be the scavenging of effete cells and cytoplasmic debris, as well as recycling of useful molecules. This is carried out by a process of heterophagic digestion using lysosomal enzymes generated in the cytoplasm of the sustentacular tissue. Conceivably, endonuclease-type enzymes feature in the lysosomal arsenal of the sustentacular tissue, and they may well be responsible for generating DNA strand breaks in target cells and nuclear debris scavenged from the dysfunctional spermatogenetic elements in the abnormal testis tubules of Cullompton and Sligo Type 1 flukes. Presumably also, the sustentacular tissue may have a role in scavenging cells damaged by TCBZ action. Thus, in the testis profiles of fluke sections strongly labelled by the TUNEL reaction, heterophagic activity in the sustentacular tissue of fluke testis tubules may augment the positive signal generated by internal endonucleases within apoptotic germinal-line cells. This may explain the widespread general labelling over the eosinophilic extracellular tubule contents in testis profiles of treated TCBZ-S flukes, as oligonucleotide

fragments, generated by heterophagic activity in the sustentacular tissue, diffuse into the adjacent seminal fluid.

In sections of the ovarian tubules of TCBZ-S Cullompton isolate flukes exposed to TCBZ metabolites *in vivo*, oogonia and primary oocytes that showed morphological features suggestive of apoptosis also labelled strongly and discretely with the TUNEL reagent. In this tissue, no evidence of heterophagic activity has been reported, although a layer of nurse cells, believed to have supportive and nutritive functions for the germinal-line cells, is interposed between the latter and the ovarian wall (Bjorkman and Thorsell, 1964; Gresson, 1964). Therefore, the labelling pattern in the ovarian tubules of TCBZ-treated sensitive flukes probably reflects apoptosis in oogonia attempting to initiate mitosis, and oocytes in the initial stages of the first meiotic division. Primary oocytes move out of the ovary at the end of prophase of the first meiotic division, completing their development in the proximal coils of the uterus (Gresson, 1964). In the triploid Cullompton isolate flukes, development of the ova appears to proceed parthenogenetically without a reductive division, so in untreated Cullompton flukes apoptosis in the oocytes is not triggered by abortive attempts at meiosis, unlike the situation with primary spermatocytes (Hanna et al., 2008).

In the vitelline tissue of TCBZ-S flukes, *in vivo* exposure to TCBZ resulted in the arrest of mitosis in stem cells at the periphery of each follicle. Thereafter, there was progressive depletion of the cell population as pre-existing vitelline cells moved through, firstly, synthesis of shell protein globules, and finally glycogen accumulation, before moving away from the follicle (as described by Irwin and Threadgold, 1970; Threadgold, 1982). At 48 and 72h post-treatment, many of the peripherally-located stem cells displayed morphological changes consistent with apoptosis (rounding, isolation, pyknosis, cytoplasmic eosinophilia) and their apoptotic state was confirmed by heavy and discrete labelling with the TUNEL reagent. As in the ovary, labelling was largely confined to the dividing cells. A network of cytoplasmic nurse cell extensions surrounds supports and nourishes the developing vitelline cells (Irwin and Threadgold, 1970) but, apart from occasional indications of circumferential extension of TUNEL labelling at the periphery of

the follicles, there was little evidence to suggest that significant endonuclease activity was associated with them.

In those tissues of TCBZ-S flukes where cell division was not prerequisite to physiological function (Mehlis' gland, uterus, tegument, gut, parenchyma, etc.) no evidence of TUNEL labelling was seen and, while *in vivo* exposure to TCBZ certainly results in the development of histological and ultrastructural abnormalities (reviewed by Fairweather and Boray, 1999; Fairweather, 2009, 2011b; Hanna et al., 2010; Toner et al., 2010a, b), these changes are not consistent with apoptosis. Perhaps in these non-germinal cell types, TCBZ action is at cytoplasmic rather than nuclear level, and hence less liable to trigger the cascade of molecular events leading to caspase activation and apoptosis (Kumar et al., 2005). Microtubule inhibition has previously been evoked to account for ultrastructural changes that develop in the tegument of TCBZ-treated sensitive flukes (Fairweather, 2005, 2009, 2011b).

Sections of TCBZ-treated resistant Sligo isolate *F. hepatica* did not exhibit any of the morphological changes characteristic of apoptosis in the testis, ovary, vitelline follicles or elsewhere in the reproductive tract or somatic tissues, supporting the histological findings of Hanna et al. (2010). Apart from the irregular positive labelling seen in the peripheral zone of the testis tubules in untreated and TCBZ-treated Sligo Type 2 flukes, accounted for by heterophagy in the sustentacular tissue (section 4.3), no positive TUNEL labelling was seen over any of the reproductive or somatic tissues. Likewise, in flukes from the three field cases where TCBZ resistance was initially suspected by the flock owner, and subsequently confirmed by FEC reduction and coproantigen reduction, no morphological changes indicative of apoptosis in the reproductive organs were seen. Furthermore, TUNEL labelling gave negative results for all tissues.

In contrast, sections of flukes from TCBZ-treated rats that had been infected with metacercariae collected from premises with no history of TCBZ resistance displayed morphological changes indicative of apoptosis in the testis tubules, ovary and vitelline follicles. This interpretation was supported by strong positive TUNEL labelling in the same tissues, distributed in patterns analogous to those described for treated TCBZ-S Cullompton flukes.

While the histological and immunocytochemical examination of sections of TCBZ-treated flukes from field cases of fasciolosis clearly cannot replace the use of FECRT and CRT for cheap and rapid diagnosis of TCBZ resistance, the former approach has the potential to provide supporting evidence for the TCBZ resistance status of fluke infections prevalent in individual flocks, and may obviate the need for follow-up by expensive and time-consuming field trials. In the current work, the demonstration of apoptosis by *in situ* hybridisation in TCBZ-S flukes, and not in TCBZ-R flukes, was descriptive and qualitative. As an adjunct to diagnosis of the TCBZ resistance status in field situations, a quantitative approach would be preferable, and this might be achieved by the use of an 'image analysis' programme. This would enable statistical comparison of results, and might prove particularly interesting in cases where TCBZ resistance was partial.

Acknowledgements

We gratefully acknowledge the co-operation and expertise of the staff of the Histopathology Section, the Pathology Section and the Photography Unit, AFBI. Certain elements of this work were carried out under the auspices of the EU Sixth Framework DELIVER programme, grant no. food-CT-200X-023025.

References

- Bjorkman, N., Thorsell, W., 1964. On the ultrastructure of the ovary of the liver fluke (*Fasciola hepatica* L.). Z. Zellforsch. Mikrosk. Anat. 63, 538–549.
- Boray, J.C., Crowfoot, P.D., Strong, M.B., Allison, J.R., Schellenbaum, M., Von Orelli, M., Sarasin, G., 1983. Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole. Vet. Rec. 113, 315–317.

- 580 Cohen, G.M., Sun, X.M., Snowden, R.T., Dinsdale, D., Skilleter, D.N., 1992.
 581 Key morphological features of apoptosis may occur in the absence of
 582 internucleosomal DNA fragmentation. *Biochem. J.* 286, 331–334.
 583
- 584 Compton, M.M., 1992. A biochemical hallmark of apoptosis: Internucleosomal
 585 degradation of the genome. *Cancer Metast. Rev.* 11, 105–119.
 586
- 587 Devine, C., Brennan, G.P., Lanusse, C.E., Alvarez, L.I., Trudgett, A., Hoey,
 588 E., Fairweather, I., 2011. Enhancement of triclabendazole action against a
 589 triclabendazole-resistant isolate of *Fasciola hepatica* by co-treatment with
 590 ketoconazole. *Vet. Parasitol.* 177, 305–315.
 591
- 592 Duvall, E., Wyllie, A.H., 1986. Death and the cell. *Immunol. Today* 7, 115–
 593 119.
 594
- 595 Fairweather, I., Boray, J.C., 1999. Mechanisms of fasciolicide action and drug
 596 resistance in *Fasciola hepatica*. In: Dalton, J.P. (Ed.), *Fasciolosis*. CABI
 597 Publishing, Wallingford, Oxon, UK, (Chapter 7), pp. 225–276.
 598
- 599 Fairweather, I., 2005. Triclabendazole: new skills to unravel an old(ish)
 600 enigma. *J. Helminthol.* 79, 227–234.
 601
- 602 Fairweather, I., 2009. Triclabendazole progress report, 2005–2009: an
 603 advancement of learning? *J. Helminthol.* 83, 139–150.
 604
- 605 Fairweather, I., 2011a. Liver fluke isolates: a question of provenance. *Vet.*
 606 *Parasitol.* 176, 1–8.
 607
- 608 Fairweather, I., 2011b. Reducing the future threat from (liver) fluke: realistic
 609 prospect or quixotic fantasy? *Vet. Parasitol.* 180, 133–143.
 610
- 611 Fairweather, I., 2011c. Raising the bar on reporting ‘triclabendazole
 612 resistance’. *Vet. Rec.* 168, 514–515.

- 613
 614 Fairweather, I., McShane, D.D., Shaw, L., Ellison, S.E., O'Hagan, N.T., York,
 615 E.A., Trudgett, A., Brennan, G.P. 2012. Development of an egg hatch assay
 616 for the diagnosis of triclabendazole resistance in *Fasciola hepatica*: proof of
 617 concept. *Vet. Parasitol.* 183, 249-259.
- 618
 619 Flanagan, A.M., Edgar, H.W.J., Forster, F., Gordon, A., Hanna, R.E.B.,
 620 McCoy, M., Brennan, G.P., Fairweather, I., 2011a. Standardisation of a
 621 coproantigen reduction test (CRT) protocol for the diagnosis of resistance to
 622 triclabendazole in *Fasciola hepatica*. *Vet. Parasitol.* 176, 34–42.
- 623
 624 Flanagan, A.M., Edgar, H.W.J., Gordon, A., Hanna, R.E.B., Brennan, G.P.,
 625 Fairweather, I., 2011b. Comparison of two assays, a faecal egg count
 626 reduction test (FECRT) and a coproantigen reduction test (CRT), for the
 627 diagnosis of resistance to triclabendazole in *Fasciola hepatica* in sheep. *Vet.*
 628 *Parasitol.* 176, 170–176.
- 629
 630 Fletcher, H.L., Hoey, E.M., Orr, N., Trudgett, A., Fairweather, I., Robinson,
 631 M.W., 2004. The occurrence and significance of triploidy in the liver fluke,
 632 *Fasciola hepatica*. *Parasitology* 128, 69–72.
- 633
 634 Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A., 1992. Identification of
 635 programmed cell death in situ via specific labeling of nuclear DNA
 636 fragmentation. *J. Cell Biol.* 119, 493-501.
- 637
 638 Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H.-P., Toyka,
 639 K.V., Lassmann, H., 1994. Differentiation between cellular apoptosis and
 640 necrosis by the combined use of in situ tailing and nick translation techniques.
 641 *Lab. Invest.* 71, 219-225.
- 642
 643 Gorczyca, W., Bigman, K., Mittelman, A., Ahmed, T., Gong, J., Melamed,
 644 M.R., Darzynkiewicz, Z., 1993. Induction of DNA strand breaks associated
 645 with apoptosis during treatment of leukemia. *Leukemia* 7, 659-670.

- 646
 647 Gresson, R.A.R., 1964. Electron microscopy of the ovary of *Fasciola hepatica*.
 648 Quart. J. Microsc. Sci. 105, 213–218.
 649
- 650 Hanna, R.E.B., Edgar, H., Moffett, D., McConnell, S., Fairweather, I.,
 651 Brennan, G.P., Trudgett, A., Hoey, E.M., Cromie, C., Taylor, S.M., Daniel, R.,
 652 2008. *Fasciola hepatica*: histology of the testis in egg-producing adults of
 653 several laboratory-maintained isolates of flukes grown to maturity in cattle and
 654 sheep and in flukes from naturally infected hosts. Vet. Parasitol. 157, 222–
 655 234.
 656
- 657 Hanna, R.E.B., Edgar, H.W.J., McConnell, S., Toner, E., McConville, M.,
 658 Brennan, G.P., Devine, C., Flanagan, A., Halferty, L., Meaney, M., Shaw, L.,
 659 Moffett, D., McCoy, M., Fairweather, I., 2010. *Fasciola hepatica*: histological
 660 changes in the reproductive structures of triclabendazole (TCBZ)-sensitive
 661 and TCBZ-resistant flukes after treatment *in vivo* with TCBZ and the related
 662 benzimidazole derivative, Compound Alpha. Vet. Parasitol. 168, 240–254.
 663
- 664 Hanna, R.E.B., Scarcella, S., Solana, H., McConnell, S., Fairweather, I.,
 665 2012a. Early onset of changes to the reproductive system of *Fasciola*
 666 *hepatica* following *in vivo* treatment with triclabendazole. Vet. Parasitol. 184,
 667 341–347.
 668
- 669 Hanna, R.E.B., Moffett, D., Brennan, G.P., Fairweather, I., 2012b. *Fasciola*
 670 *hepatica*: a light and electron microscope study of sustentacular tissue and
 671 heterophagy in the testis. Vet. Parasitol. In press, (doi number
 672 10.1016/j.vetpar.2011.12.037).
 673
- 674 Hennessy, D.R., Lacey, E., Steel, J.W., Prichard, R.K., 1987. The kinetics of
 675 triclabendazole disposition in sheep. J. Vet. Pharmacol. Ther. 10, 64–72.
 676

- 677 Irwin, S.W.B., Threadgold, L.T., 1970. Electron microscope studies on
678 *Fasciola hepatica*. VIII. The development of the vitelline cells. Exp. Parasitol.
679 28, 399-411.
- 680
- 681 Kumar, V., Abbas, A.K., Fausto, N., 2005. Robbins and Cotran Pathologic
682 Basis of Disease, seventh ed. Elsevier Saunders, Philadelphia, Pennsylvania,
683 USA, pp 26–32.
- 684
- 685 Lacey, E., 1988. The role of the cytoskeletal protein, tubulin, in the mode of
686 action and mechanism of drug resistance to benzimidazoles. Int. J. Parasitol.
687 8, 399-404.
- 688
- 689 McConville, M., Brennan, G.P., Flanagan, A., Edgar, H.W.J., Hanna, R.E.B.,
690 McCoy, M., Gordon, A.W., Castillo, R., Hernández-Campos, A., Fairweather
691 I., 2009. An evaluation of the efficacy of compound alpha and triclabendazole
692 against two isolates of *Fasciola hepatica*. Vet. Parasitol. 162, 75-88.
- 693
- 694 Olaechea, F., Lovera, V., Larroza, M., Raffo, F., Cabrera, R., 2011.
695 Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia
696 (Argentina). Vet. Parasitol. 178, 364-366.
- 697
- 698 Overend, D.J., Bowen, F.L., 1995. Resistance of *Fasciola hepatica* to
699 triclabendazole. Aust. Vet. J. 72, 275–276.
- 700
- 701 Threadgold, L.T. 1982. Stereological analysis of vitelline cell development.
702 Exp. Parasitol. 54, 352-365.
- 703
- 704 Toner, E., Brennan, G.P., Hanna, R.E.B., Edgar, H.W.J., Fairweather, I.,
705 2010a. Tegumental surface changes in adult *Fasciola hepatica* in response to
706 treatment *in vivo* with triclabendazole in the sheep host. Vet. Parasitol. 172,
707 238–248.
- 708

Toner, E., Brennan, G.P., Hanna, R.E.B., Edgar, H.W.J., Fairweather, I.,
2010b. Time-dependent changes to the tegumental system and gastrodermis
of adult *Fasciola hepatica* following treatment *in vivo* with triclabendazole in
the sheep host. Vet. Parasitol. 174, 218-227.

Toner, E., Brennan, G.P., Hanna, R.E.B., Edgar, H.W., Fairweather, I., 2011a.
Disruption of egg formation by *Fasciola hepatica* following treatment *in vivo*
with triclabendazole in the sheep host. Vet. Parasitol. 177, 79-89.

Toner, E., Brennan, G.P., Hanna, R.E.B., Edgar, H.W.J., Fairweather, I.,
2011b. *Fasciola hepatica*: time-dependent disruption of spermatogenesis
following *in vivo* treatment with triclabendazole. Parasitol. Res. 109, 1035–
1043.

Description of figures.

Fig. 1.

(a) *F. hepatica*, TCBZ-S Cullompton isolate, untreated, H&E stain.

Clusters of primary and secondary spermatogonia (Sg1/2) occur at the
periphery of the testis tubules (Te), while the most abundant cells
present are tertiary spermatogonia (Sg3), which occupy most of the
remaining space in the tubules. Abnormal primary spermatocytes
(Sp1), often exhibiting pyknosis or karyorrhexis, are evident, but no
secondary spermatocytes, spermatids or spermatozoa occur in flukes
of this isolate. Occasional rounded eosinophilic bodies containing
multiple dense nuclear fragments and probably representing apoptotic
spermatocytes, occur throughout the tubules, often in peripheral
vacuoles (arrow). T = tegumental syncytium; G = gut;

P = parenchyma.

(b) *F. hepatica*, TCBZ-S Cullompton isolate, untreated, TUNEL reaction.

Brown reaction product indicating sites of endonuclease-induced DNA
strand breaks is associated with occasional large bodies (arrowed) that
are mainly located in peripheral vacuoles of the testis tubules (Te).

740 These apoptotic bodies correspond to rounded eosinophilic
 741 spermatocytes. G = gut; T = tegumental syncytium; Tc = tegumental
 742 cell bodies.

743 (c) *F. hepatica*, TCBZ-S Cullompton isolate, untreated, TUNEL, negative
 744 control. No labelling is present over the testis tubules (Te), tegument
 745 (T), gut (G), parenchyma (P) or elsewhere.

746 (d) *F. hepatica*, TCBZ-S Cullompton isolate, untreated, TUNEL, positive
 747 control. All nuclei in the section are labelled, including those of the
 748 testis (Te), gut (G), parenchyma (P) and tegumental cell bodies (Tc).
 749 The positive signal was generated by applying exogenous nuclease to
 750 the sections prior to labelling.

751

752 Fig. 2.

753 (a) *F. hepatica*, TCBZ-S Cullompton isolate, 48h TCBZ-treated, H&E stain.
 754 The testis tubules (Te) are rather depleted of cells. Numerous rounded
 755 eosinophilic bodies (arrows) and pyknotic mononuclear cells are
 756 surrounded by eosinophilic hyaline material, probably fluid (F), and also
 757 occur in the peripheral vacuoles (V) of the testis tubules. Primary and
 758 secondary spermatogonia (Sg1/2) are evident at the periphery of the
 759 tubules. G = gut; T = tegumental syncytium.

760 (b) *F. hepatica*, TCBZ-S Cullompton isolate, 48h TCBZ-treated, TUNEL
 761 reaction. Strong positive labelling is evident throughout the testis
 762 profiles (Te), but particularly over the rounded dense apoptotic
 763 spermatocytes and spermatogonia (arrowed) which are abundant in
 764 the vacuoles (V). The fluid content (F) of the tubules is also labelled,
 765 but no reaction product is present over the nuclei of the tegumental cell
 766 bodies (Tc), the gut (G) or the parenchyma (P).

767 (c) *F. hepatica*, TCBZ-S Cullompton isolate, untreated, TUNEL reaction. In
 768 a profile of an ovarian tubule (Ot), oogonia with condensed
 769 heterochromatic nuclei (Og) occupy the peripheral zone, while
 770 spheroidal or polygonal primary oocytes (Oc) with abundant cytoplasm

771 and euchromatic nuclei bearing 1 or 2 nucleoli densely pack the core.
 772 Eggs in the uterus (U) are unlabelled, although the natural brown-
 773 yellow colour of the tanned egg shells (Es) is evident.

774 (d) *F. hepatica*, TCBZ-S Cullompton isolate, 48h TCBZ-treated, TUNEL
 775 reaction. The profiles of the ovarian tubules (Ot) are rather shrunken,
 776 due mainly to loss of oocytes. Dense labelling indicating sites of
 777 endonuclease-induced DNA strand breaks is associated with rounded
 778 apoptotic oocytes and oogonia (arrowed). No specific labelling is
 779 associated with the tegument (T), gut (G) or parenchyma (P).

780

781 Fig. 3.

782 (a) *F. hepatica*, TCBZ-S Cullompton isolate, untreated, TUNEL reaction. In
 783 the vitelline follicles (Vf), cells at all stages of development are
 784 represented. Towards the periphery of each follicle, stem cells (Vs) are
 785 present. Close to them are located intermediate cells (Vi) engaging in
 786 the synthesis of refringent shell protein globules. Towards the centre of
 787 each follicle, but often bulging to the periphery, the mature vitelline
 788 cells (Vm), swollen with stored glycogen and featuring marginalised
 789 shell protein globule clusters (Vg), are evident. All cells in the follicles
 790 are unlabelled, as in the gut (G) and tegument (T).

791 (b) *F. hepatica*, TCBZ-S Cullompton isolate, 48h TCBZ-treated, TUNEL
 792 reaction. The vitelline follicles (Vf) are rather shrunken and deficient in
 793 stem cells and intermediate vitelline cells. Mature cells (Vm)
 794 predominate in each and every follicle. In these cells, the shell globule
 795 clusters are often irregular in size and distribution, with loss of clear
 796 marginalisation. Brown reaction product (arrows) specifically labels
 797 apoptotic cells at the periphery of the follicles, extending
 798 circumferentially in some locations. The gut (G) and tegumental cell
 799 bodies (Tc) are unlabelled.

800 (c) *F. hepatica*, TCBZ-S Cullompton isolate, 48h TCBZ-treated, TUNEL
 801 reaction. The Mehlis' gland complex comprises two types of secretory

cells (S1 and S2) which have elongated cytoplasmic connections (Mc) to the ootype. Coils of the proximal uterus (Up) are also embedded in the glandular tissue. A profile of Laurer's canal (L) is partially surrounded by the dorsal cells of the mass. The cytoplasm of the gland cells, the connecting ducts and the supporting parenchymal tissue appear vesiculated and shrunken, but there is no labelling indicative of apoptotic change. Profiles of uterus (U), gut (G) and tegument (T) in the section are unlabelled, but, in comparison, dense labelling is associated with a testis tubule (Te).

(d) *F. hepatica*, TCBZ-S Cullompton isolate, 48h TCBZ-treated, TUNEL reaction. Profiles of the uterus (U) contain no shelled eggs, but numerous mature vitelline cells (Vm), occasional oocytes (Oc), and irregular masses of shell protein material (Vg) that have been discharged from the vitelline cells. The uterus and its contents are unlabelled, but the tanned shell protein material has a natural pale yellow-brown colour, readily distinguishable from the dense brown reaction product labelling the testis profiles (Te). Gut (G), and parenchyma (P) are unlabelled.

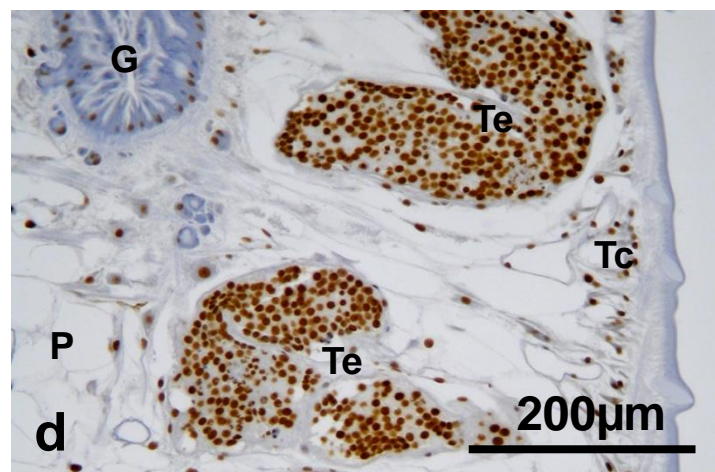
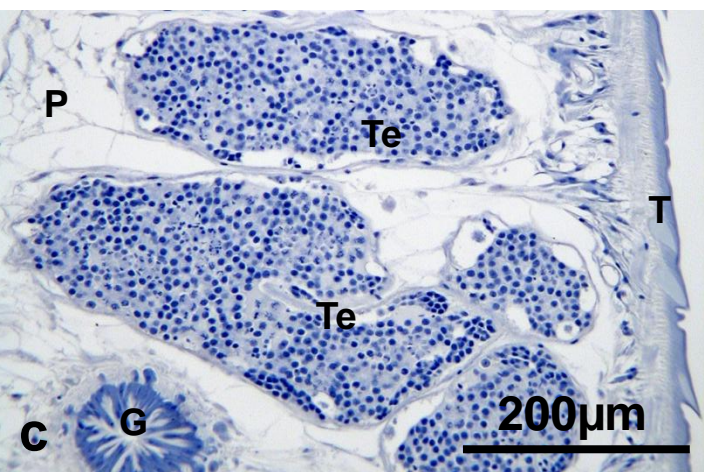
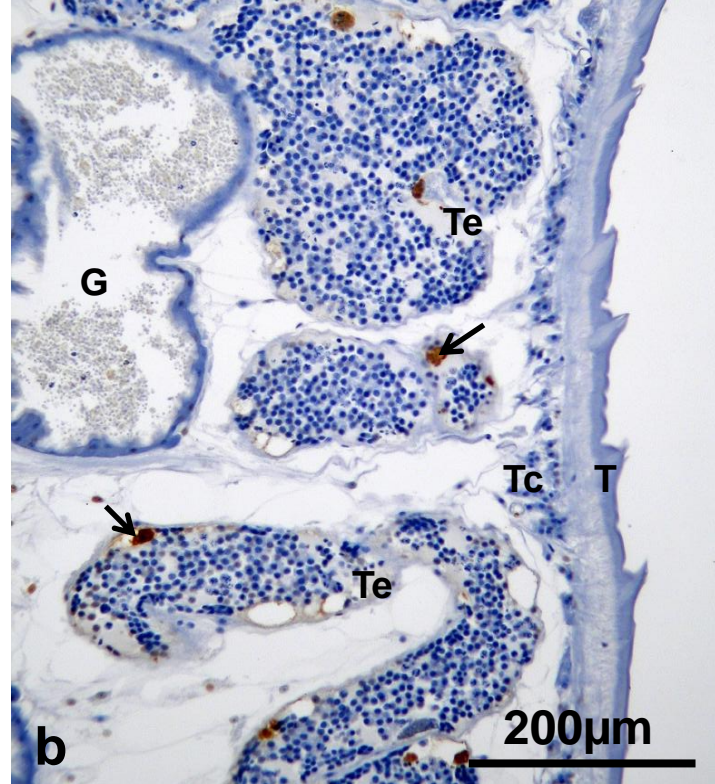
(e) *F. hepatica*, TCBZ-S Cullompton isolate, untreated, TUNEL reaction. In a profile of the uterus (U), fully-formed eggs, each with an intact yellow-brown tanned shell (Es) are evident. The structures are unlabelled. G = gut.

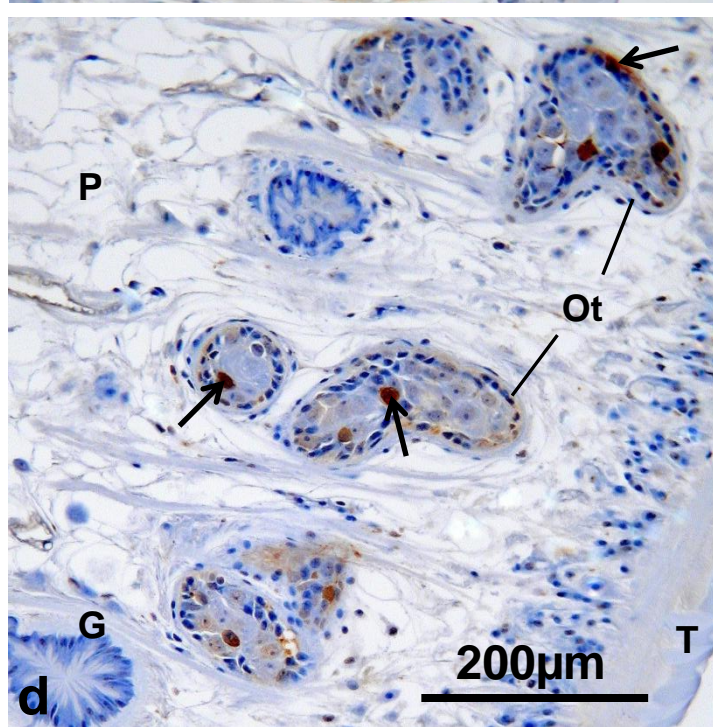
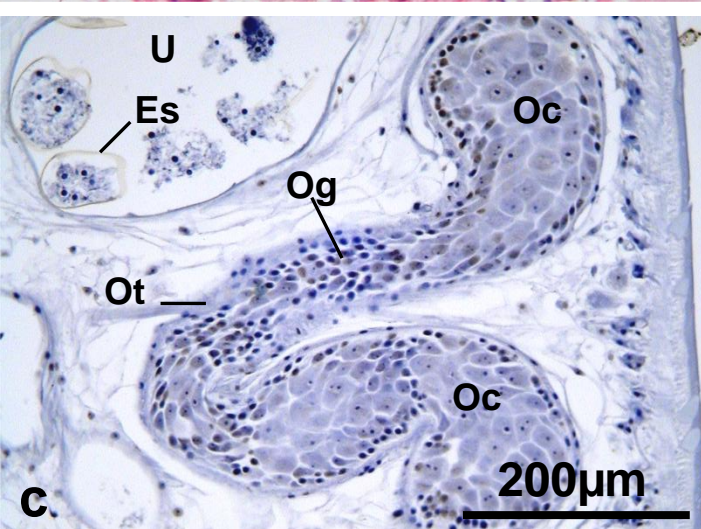
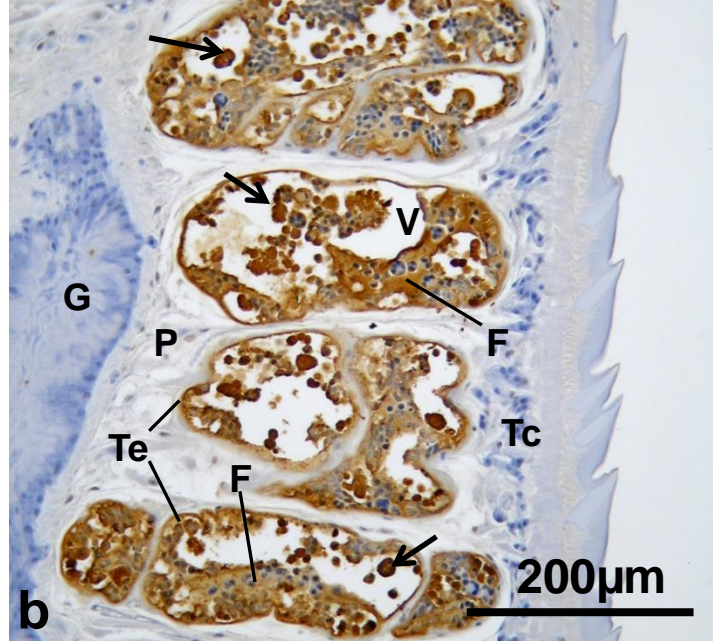
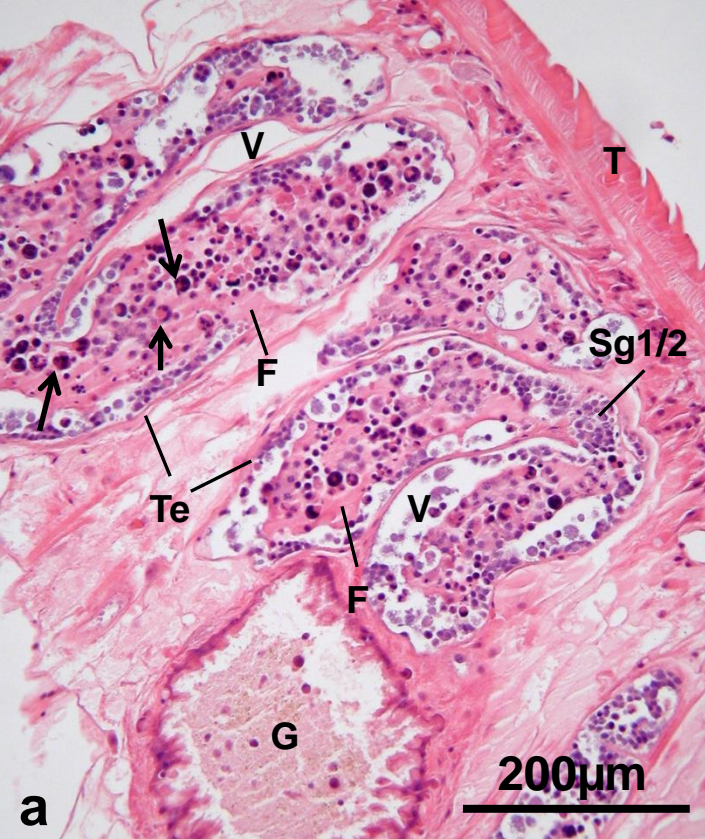
Fig.4.

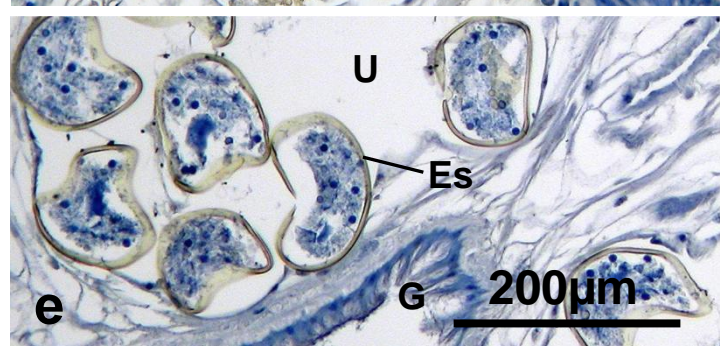
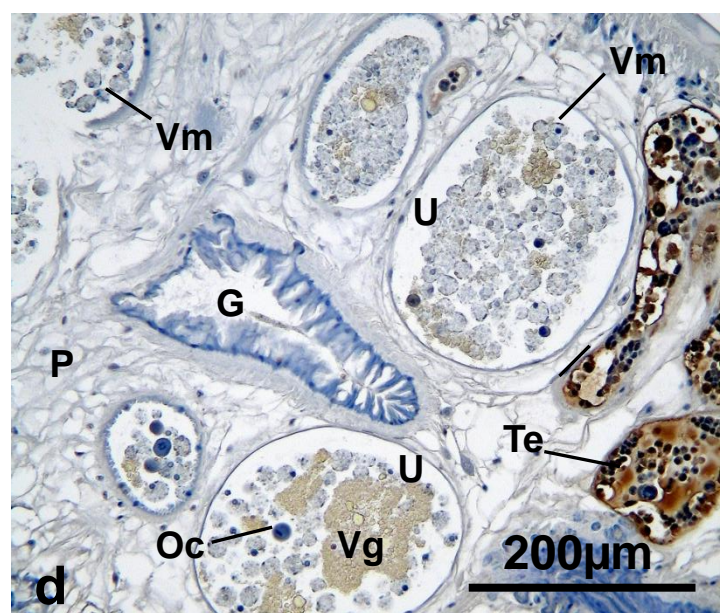
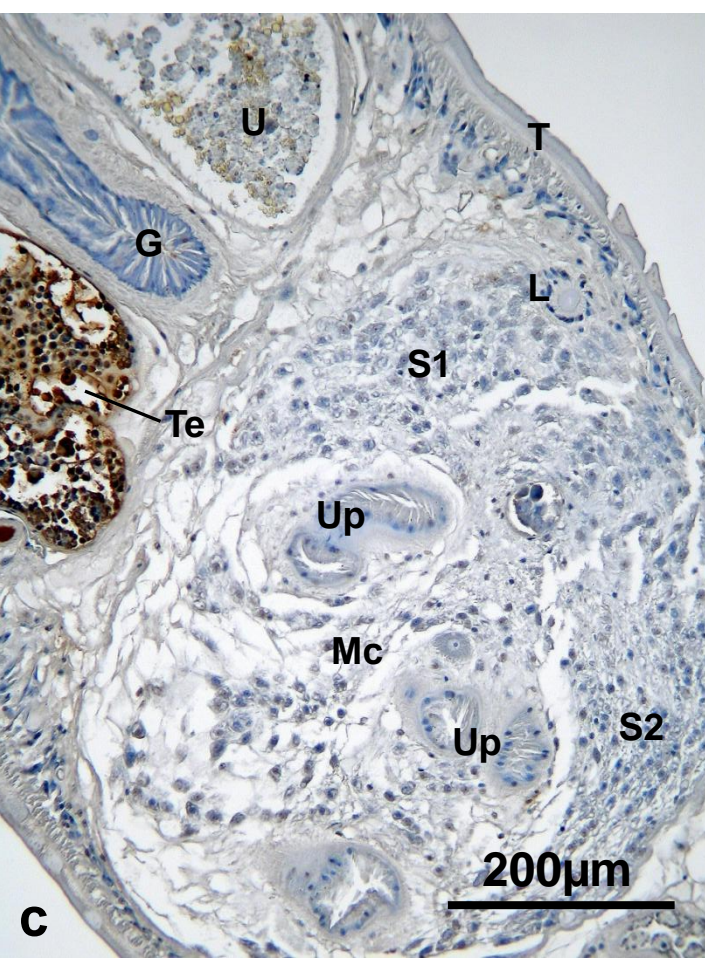
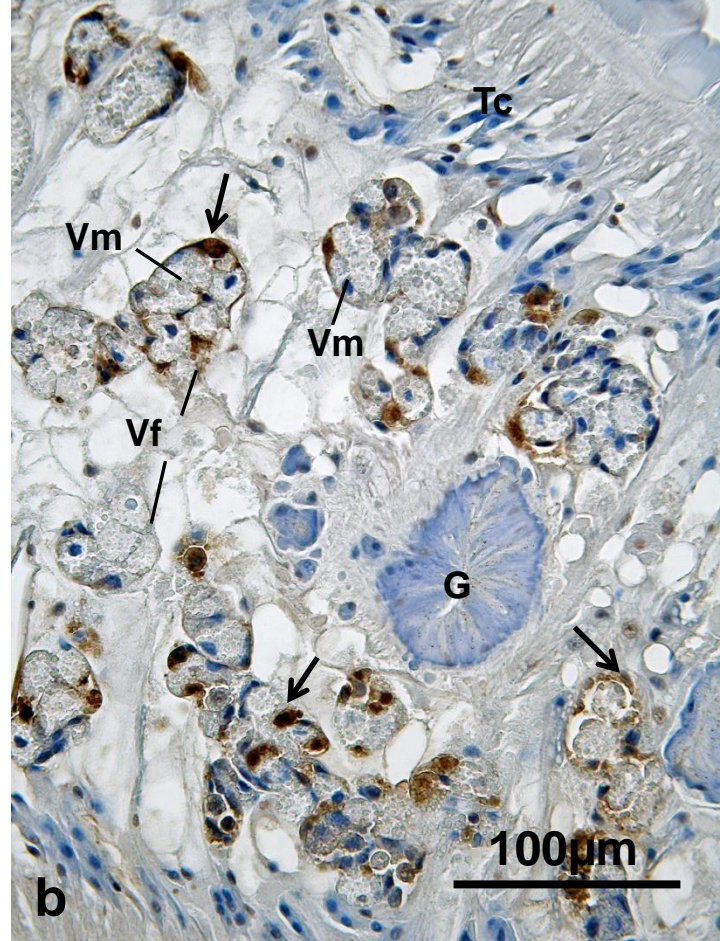
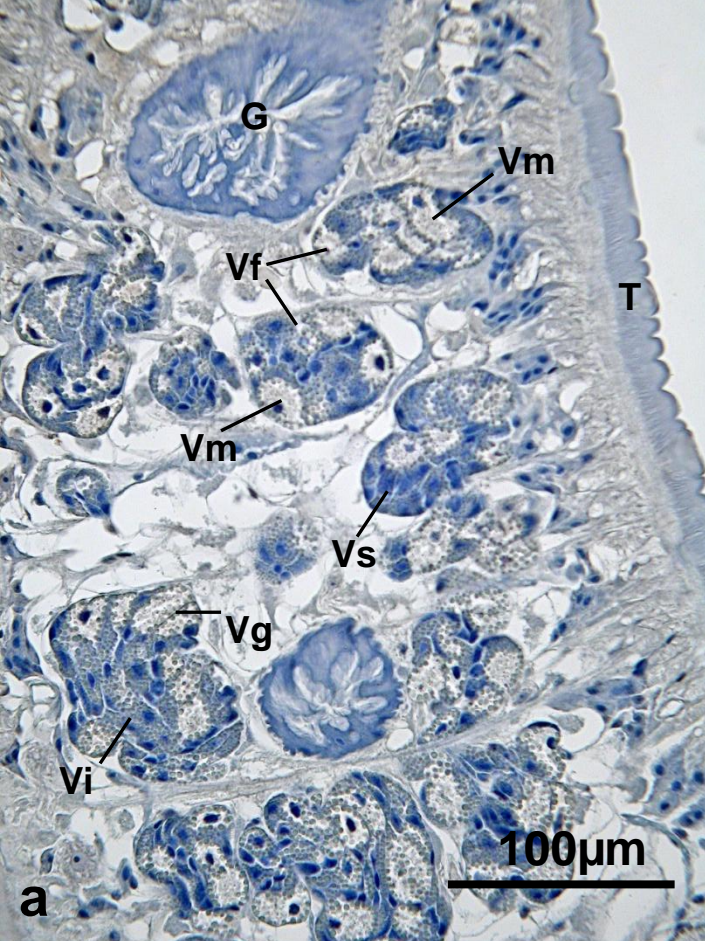
(a) *F. hepatica*, TCBZ-R Sligo Type 1 isolate, 48h TCBZ-treated, TUNEL reaction. The testis tubules (Te) contain primary, secondary and tertiary spermatogonia (Sg1/2, Sg3 respectively), spermatocytes (Sc), spermatids (Sp) and mature spermatozoa (Sz). There is no evidence of TCBZ-induced histological change, and no labelling indicative of endonuclease-induced DNA strand breaks associated with apoptosis.

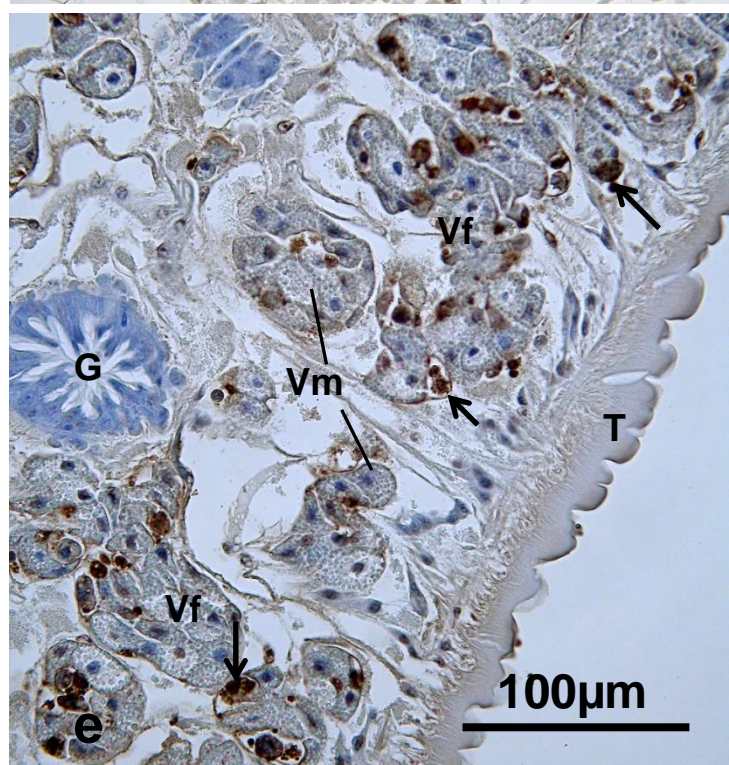
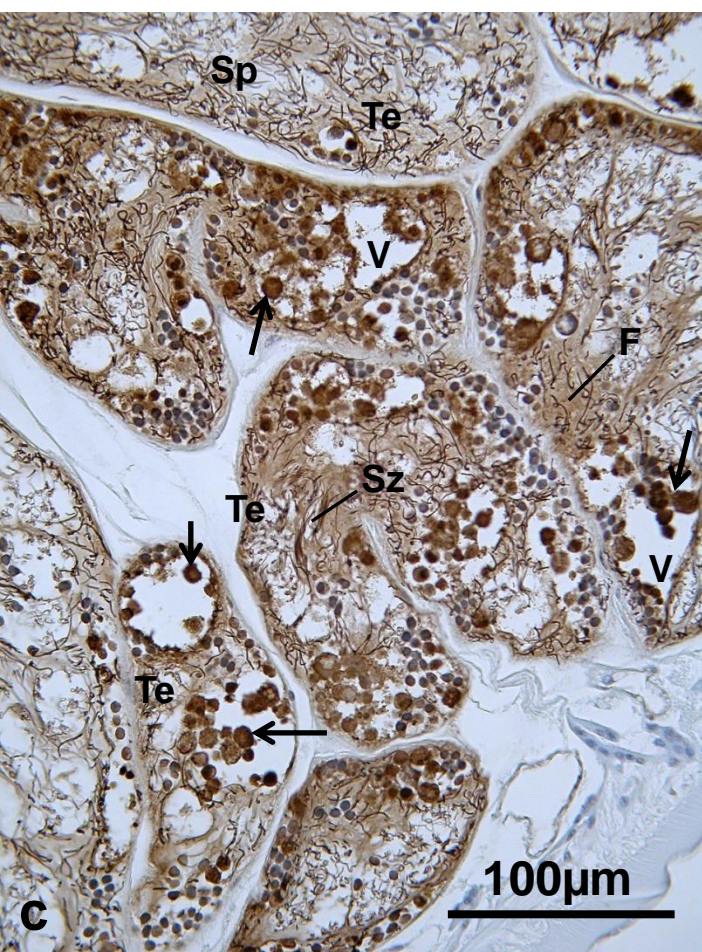
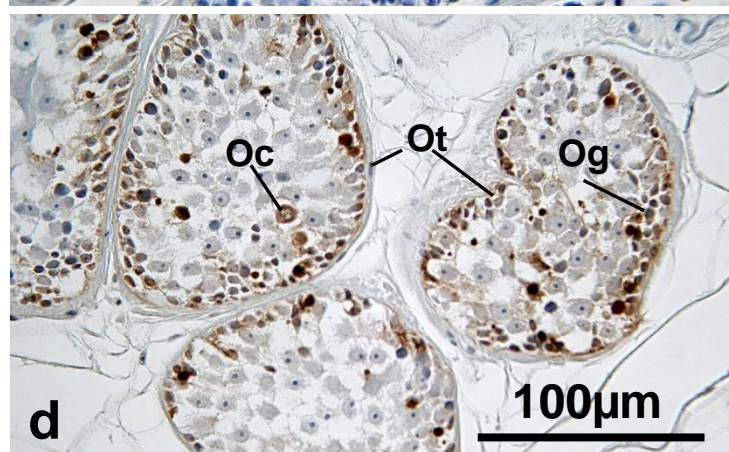
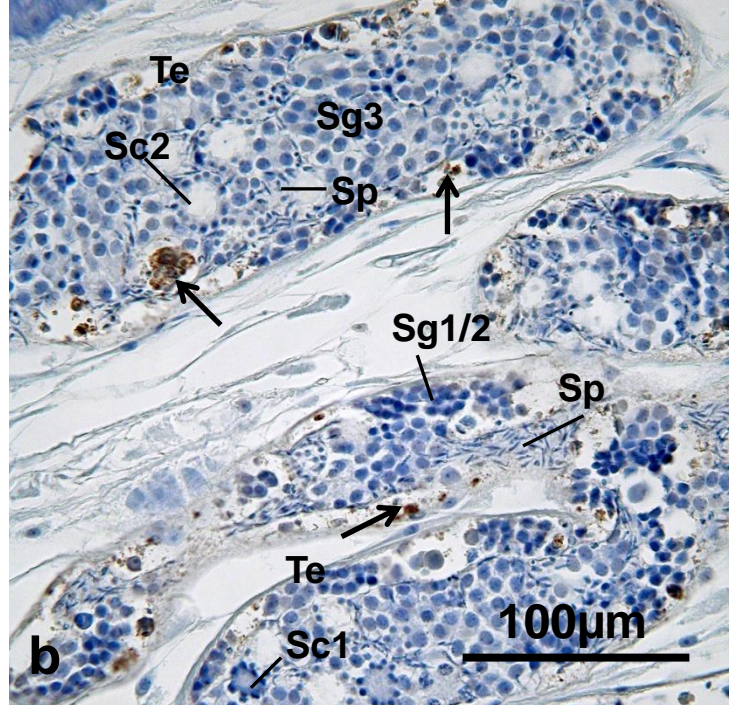
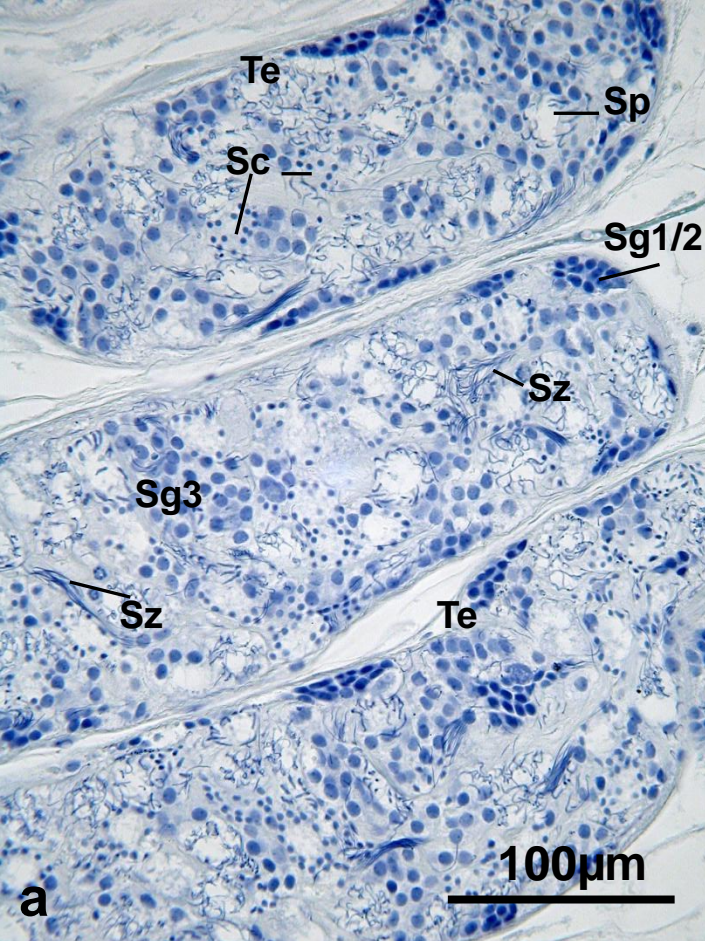
- (b) *F. hepatica*, TCBZ-R Sligo Type 2 isolate, untreated, TUNEL reaction. The testis tubules (Te) contain spermatogonia (Sg1/2, Sg3), spermatocytes (Sc1, Sc2) and elongating spermatids (Sp), but no mature spermatozoa. Peripherally, there is vacuolation, with irregularly-sized granules and bodies showing positive reactivity for endonuclease-induced DNA strand breaks (arrowed).
- (c) *F. hepatica*, TCBZ-S Bovine field isolate, 48h TCBZ-treated, TUNEL reaction. The profiles of testis (Te) are rather shrunken and depleted of cells, showing marked peripheral vacuolation (V), and a relative increase in fluid content. Numerous rounded spermatocytes and tertiary spermatogonia (arrowed) lying in the peripheral vacuoles and in the core region of the tubules are densely labelled for endonuclease-induced DNA strand breaks, indicating apoptosis. The hyaline fluid shows moderately intense labelling (F). Spermatids (Sp) and mature spermatozoa (Sz) are visible in the sections.
- (d) *F. hepatica*, TCBZ-S Bovine field isolate, 48h TCBZ-treated, TUNEL reaction. The ovarian tubules (Ot), which are generally rather shrunken and vacuolated, contain condensed and rounded oogonia (Og) and oocytes (Oc); many of these cells are densely labelled for endonuclease-induced DNA strand breaks, indicating apoptosis.
- (e) *F. hepatica*, TCBZ-S Bovine field isolate, 48h TCBZ-treated, TUNEL reaction. In the vitelline follicles (Vf), mature vitelline cells (Vm), which are filled with pale-staining glycogen, predominate. Stem cells and intermediate vitelline cells are reduced in number, and the dense focal staining (arrowed) at the periphery of many follicles denotes that cells in this region are undergoing apoptosis. G = gut; T = tegument.

Figure









Figure

